

	10	20	30	40	50	60	70	80	
GFP wt	MSKGEELFTGVVPIVLVDGDVNGHKFSVSGEGEDATYGKLTCLKFICTTGKLPVPWPTLVTTFSYGVQCFSRYPDHMKQ								80
EGFP	T.....LT.....								80
shGFP1	T.....E.....LT.....								80
NF_hGFP	T.....T...K.....E.....LT.....R								80
sfGFP	.....R.....N.....LG.....R								80
shGFP2	T.....T...K.....R.....E.....LT.....R								80
sinGFP1	T.....T...K.....K.....E.....LT.....K.....K								80
sinGFP4a	T.....T...K.....K.....E.....LT.....AK.....K								80
	90	100	110	120	130	140	150	160	
GFP wt	HDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIMADKQKNG								160
EGFP	.....								160
shGFP1	.....E.....R.E.....								160
NF_hGFP	.....E.....N.....R.E.....								160
sfGFP	.....S...T.....F.....T.....								160
shGFP2	.....E...T.....N.....H...R.E.....								160
sinGFP1	.....E...T...K.....K...N.....H...K.E.....								160
sinGFP4a	.....T...E...T...K.....K...D.....H...D.K.E...E...								160
	170	180	190	200	210	220	230		
GFP wt	IKVNFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNEKRDHMLLFEVTAAGITHGMDELYK								238
EGFP	.....K.....L.....								238
shGFP1	.....K.....K.....K.E..RD.								238
NF_hGFP	.....Q.E...K.....Q...N.....K.....K.E..RD.								238
sfGFP	..A.....V.....V.....								238
shGFP2	..A.....V...Q.E...K.....R.....TT.....K.....K.E..RD.								238
sinGFP1	..A...K.V...Q.E...K.....K.....TT.....K...K.....K.E..KD.								238
sinGFP4a	..A...K.V...E.E...K.E.....K...T...TT.....K...K.T.....K.E..KD.								238

### Sequence alignment A: Design of super-inert GFP variants

Figure shows sequence alignment of the key variants. Starting point was EGFP (enhanced GFP; Mut1 in Cormack et al., 1996) carrying an additional dimer-suppressing L221K mutation (Zacharias et al., 2002). shGFP1 (superhydrophilic GFP 1) combines eight mutations of hydrophobic residues to hydrophilic ones. NF\_hGFP was an attempt to make GFP even more hydrophilic, however, this variant was non-fluorescent when expressed at 37°C and nearly non-fluorescent at 18°C. Random mutagenesis and selection identified numerous fluorescence-enhancing mutations, the most hydrophilic of which as well as the dominating N200Y reversion were accepted and combined into the shGFP2 variant, which passed NPCs already ≈ 7-fold slower than EGFP and 2-fold slower than mCherry.

SinGFP1 (super-inert GFP 1) was derived from shGFP2 by 10 R→K exchanges. SinGFP4a was derived (in several steps) from sinGFP1 by increasing its negative charge (by 5 units) as well as by mutating additional exposed hydrophobic residues to hydrophilic ones. SinGFP4a passes NPCs 35-fold slower than EGFP and 10-fold slower than mCherry.

S72A, Y145H, V163A, and S205T are fold-enhancing mutations of non-exposed residues. S30R, N105T, V163A and i171V are superfolder mutations (Pédrelacq et al., 2006), while S30K, Y39E, F99E, M153E, Y145H and A206T represent alternative changes at 'superfolder positions'.

	10	20	30	40	50	60	70	80	
EGFP	TSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEDATYGKLTCLKFICTTGKLPVPWPTLVTTLTYGVCFSRYPDHMKQ								80
efGFP	.....R.....H.....								80
efGFP_3W	.....R.....H.....								80
efGFP_5W	.....R.....W.....H.....								80
efGFP_8W	.....R.....W.....H.....								80
efGFP_8Y	.....R.....Y.....H.....								80
efGFP_8F	.....R.....F.....H.....								80
efGFP_8L	.....R.....L.....H.....								80
efGFP_8i	.....R.....I.....H.....								80
efGFP_8M	.....R.....M.....H.....								80

  

	90	100	110	120	130	140	150	160	
EGFP	HDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIMADKQKNG								160
efGFP	.....Y.E..A.....F.....								160
efGFP_3W	.....W.Y.E..A.....F.....								160
efGFP_5W	.....W.Y.E..A.....F..W.....								160
efGFP_8W	.....W.Y.E..A.....F..W.....								160
efGFP_8Y	.....Y.Y.E..A.....F..Y.....								160
efGFP_8F	.....F.Y.E..A.....F..F.....								160
efGFP_8L	.....L.Y.E..A.....F..L.....								160
efGFP_8i	.....I.Y.E..A.....F..I.....								160
efGFP_8M	.....M.Y.E..A.....F..M.....								160

  

	170	180	190	200	210	220	230	
EGFP	IKVNFKIRHNIEDGSLADHYQQNTPIGDGPVLLPDNHYLSTQSALS KDPNEKRDHMLKEFVTAAGITLGMDELYK							238
efGFP	..A..NT...V...E.V.....N...K.....G....							238
efGFP_3W	..AW.NT...V...E.V.....N.W.K.....G....							238
efGFP_5W	..AW.NT...V...E.V.....N.W.K.....G....							238
efGFP_8W	..AW.NT...V...E.V.W.....N.W.K..W.....W.....G....							238
efGFP_8Y	..AY.NT...V...E.V.Y.....N.Y.K..Y.....Y.....G....							238
efGFP_8F	..AF.NT...V...E.V.F.....N.F.K..F.....F.....G....							238
efGFP_8L	..AL.NT...V...E.V.L.....N.L.K..L.....L.....G....							238
efGFP_8i	..AI.NT...V...E.V.I.....N.I.K..I.....I.....G....							238
efGFP_8M	..AM.NT...V...E.V.M.....N.M.K..M.....M.....G....							238

### Sequence alignment B: Engineering of super-hydrophobic GFP variants

The aim was to obtain GFP variants that bind FG domains well enough for a rapid NPC passage. Placing eight ectopic tryptophans onto the GFP surface lead, however, to an essentially non-fluorescent variant. Seven rounds of random mutagenesis and selection identified a set of 15 second-site-mutations that made the GFP\_8W variant highly fluorescent. The new scaffold is called efGFP (for enhanced folding of hydrophobic GFPs). Several derivatives were analyzed, namely efGFP with 3, 5, or 8 tryptophans (3W, 5W, 8W) as well as with eight tyrosines (8Y), phenylalanines (8F), leucines (8L), isoleucines (8i) or methionines (8M).

	10	20	30	40	50	60	70	80	
GFP wt	MSKGEELFTGVVPIVELDGDVNGHKFSVSGEGEDATYGKLTLLKFICTTGKLPVPWPTLVTTFSYGVQCFSRYPDHMKQ								80
sfGFP	.....R.....N.....LG.....R								80
frGFP1	T.R.....R..R.....N.R..R.....R.....RR								80
sffrGFP4	T.R.....R..R.....N.R..R.....R.....LT.....RR								80
sffrGFP4 18xR→K	T.....R.....N.....LT.....R								80
sffrGFP4 25xR→K	T.....K.....N.....LT.....K.....								80
	90	100	110	120	130	140	150	160	
GFP wt	HDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEEDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIMADKQKNG								160
sfGFP	.....S.....T.....F.....T.....								160
frGFP1	...R.....S.R..T.R....R.....R...R.....R.....T..R.R..								160
sffrGFP4	.....S.R..T.R..V.R.....R.T..R.....R.....T..R.R..								160
sffrGFP4 18xR→K	.....S.....T.....V.....T.....T.....								160
sffrGFP4 25xR→K	.....S.....T..K.V.....K.....T.....T.....								160
	170	180	190	200	210	220	230		
GFP wt	IKVNFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSALSCKDPNEKRDHMLLEFVTAAGITHGMDELYK								238
sfGFP	..A.....V.....V.....								238
frGFP1	.RA..R.....R..R.....R								238
sffrGFP4	.RA..T...V.....D...T..R..R.....Q.....R								238
sffrGFP4 18xR→K	..A..T...V.....D...T.....Q.....								238
sffrGFP4 25xR→K	..A..T.K..V.....D...T.....K.....Q.....								238

### Sequence alignment C: Lys→Arg substituted GFP variants

For a nuclear transport-unrelated project, we designed a GFP variant, where all lysines had been exchanged to arginines, the rationale being to obtain a variant that remains mobile after fixation by aldehydes.

The first variant, frGFP1 (fixation-resistant GFP 1) already contained some fold-enhancing mutations from superfolder GFP (sfGFP; Pédelaq et al., 2006). However, it was non-fluorescent. frGFP1 was then evolved to a highly fluorescent derivative, sffrGFP4 (superfolder fixation-resistant GFP4), which serendipitously turned out to pass NPCs very rapidly.

The alignment also shows sffrGFP4 variants, where the initially introduced Arg-substitutions had been reverted (sffrGFP4 18xR→K) or all surface arginines had been converted to lysines (sffrGFP4 25xR→K). These crossed NPCs ~40 and ~100-fold slower, respectively.

	10	20	30	40	50	60	70	80	90	100	110	120		
EGFP			S								R	F.K.N		120
efGFP	TSKGEELFTGVVPILEVELDGDVNGHKFSVRGEGEDATYGKLTGKLFICTTGKLPVPWPTLVTTLYGVQCFSHYPDHKQHDFFKSAMPEGYVQERTIYFEDDGAYKTRAEVKFEGDTLV												120	
efGFP_8K												K	120	
efGFP_8E												E	120	
efGFP_8N												N	120	
efGFP_8S												S	120	
efGFP_8T												T	120	
efGFP_8Q												Q	120	
efGFP_8R												R	120	
efGFP_8H												H	120	
efGFP_8A												A	120	
efGFP_8L												L	120	
efGFP_8V												V	120	
efGFP_8M												M	120	
efGFP_8i												I	120	
efGFP_8C												C	120	
efGFP_8F												F	120	
efGFP_8Y												Y	120	
efGFP_8W												W	120	

	130	140	150	160	170	180	190	200	210	220	230		
EGFP			Y	V.KI		I	V.L		S.A		D		238
efGFP	NRIELKGIIDFKEDGNILGHKLEYNFNHNVYIMADKQKNGIKANFNTRHNVEDGSEQVADHYQQNTPIGDGPVLLPDNHYLNTQSKLSKDPNEKRDHMLKEFVTAAGITLGMGELYK												238
efGFP_8K												K	238
efGFP_8E												E	238
efGFP_8N												N	238
efGFP_8S												S	238
efGFP_8T												T	238
efGFP_8Q												Q	238
efGFP_8R												R	238
efGFP_8H												H	238
efGFP_8A												A	238
efGFP_8L												L	238
efGFP_8V												V	238
efGFP_8M												M	238
efGFP_8i												I	238
efGFP_8C												C	238
efGFP_8F												F	238
efGFP_8Y												Y	238
efGFP_8W												W	238

**Sequence alignment D: efGFP\_8x variants for testing which surface residues impede or facilitate FG phase-entry**

Alignment shows the GFP variants tested in Figure 4A and 4B. Each of them displays eight copies of the indicated amino acid side chain.

	10	20	30	40	50	60	70	80	
sffrGFP4	TSRGEELFTGVVPILVELDGDVNGHRFSVRGEGEGDATNGRLTLRFICTTGRLPVPWPTLVTTLTYGVCFSRYPDHMRR								80
sffrGFP5	.....			D.....					80
sffrGFP6	.....Q.....								80
sffrGFP7	.....QQ.....								80
	90	100	110	120	130	140	150	160	
sffrGFP4	HDFFKSAMPEGYVQERTISFRDDGTYRTRAVVRFEGDTLVNRIELRGTDVFREDGNILGHRLEYNYNSHNVYITADRQRNG								160
sffrGFP5	.....					D.....	D.D.....	E...	160
sffrGFP6	.....			N.....	N.....				160
sffrGFP7	.....			N.....	N.....				160
	170	180	190	200	210	220	230		
sffrGFP4	IRANFTIRHNVEDGSVQLADHYQQNTPIGDGPVLLPDDHYLSTQTALSRDPNERRDHMVLLFVTAAGITQGMDLYR								238
sffrGFP5	.....		E.....			D.....		E.....	238
sffrGFP6	.....	N.....	N.....					Q...	238
sffrGFP7	.....	N.....	N.....					NQ...	238

**Sequence alignment E: Charge-series based on sffrGFP4**

To test the effect of net charge on NPC passage without tampering with exposed arginines or hydrophobic residues, we designed a charge series based on sffrGFP4. All introduced changes were isosteric N-D or Q-E exchanges.

	10	20	30	40	50	60	70	80	
EGFP	TSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEDATYGKLT LKFICTTGKLPVPWPTLVTTLT YGVQCF SRYPDHMKQ								80
sfrrGFP4	.R.		R.	R.	N.R.	R.			RR 80
efGFP_3W			R.					H.	80
efGFP_5W			R.	W.				H.	80
efGFP_8W			R.	W.				H.	80
efGFP_8i			R.	I.				H.	80
MaxR_3W	.R.		R.	R.	N.R.	R.			RR 80
MaxR_5W	.R.		R.	R.	N.W.	R.			RR 80
MaxR_8i	.R.		R.	R.	N.I.	R.			RR 80

  

	90	100	110	120	130	140	150	160	
EGFP	HDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGDIFKEDGNILGHKLEYNYN SHNVYIMADKQKNG								160
sfrrGFP4		S.R.	T.R.	V.R.		R.T.	R.		T.R.R. 160
efGFP_3W		W.Y.E.	A.				F.		160
efGFP_5W		W.Y.E.	A.				F.	W.	160
efGFP_8W		W.Y.E.	A.				F.	W.	160
efGFP_8i		I.Y.E.	A.				F.	I.	160
MaxR_3W		W.Y.	A.	V.R.		R.T.	R.		T.R.R. 160
MaxR_5W		W.Y.	A.	V.R.		R.T.	R.		T.R.R. 160
MaxR_8i		I.Y.R.	A.R.	V.R.		R.T.	R.		I.T.R.R. 160

  

	170	180	190	200	210	220	230							
EGFP	IKVNFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSALS KDPNEKRDHMLKEFVTAAGITLGMDELYK							238						
sfrrGFP4	.RA.	T.	V.		D.	T.	R.	R.	L.	Q.	R	238		
efGFP_3W	.AW.	NT.	V.	E.V.		N.W.K.					G.	238		
efGFP_5W	.AW.	NT.	V.	E.V.		N.W.K.					G.	238		
efGFP_8W	.AW.	NT.	V.	E.V.W.		N.W.K.	W.				G.	238		
efGFP_8i	.AI.	NT.	V.	E.V.I.		N.I.K.	I.				G.	238		
MaxR_3W	.AW.	T.	V.	E.V.		D.	N.WTR.	R.	R.	L.	Q.	R	238	
MaxR_5W	.AW.	T.	V.	E.V.W.		D.	N.WTR.	R.	R.	L.	Q.	R	238	
MaxR_8i	.RAI.	T.	V.	E.V.I.		D.	N.ITR.	I.	R.	L.	I.	Q.	R	238

### Sequence alignment F: A first generation of GFP<sup>NTR</sup> variants

The sfrrGFP4 (MaxR) variant and super-hydrophobic variants are shown again for reference. MaxR\_3W, MaxR\_5W, and MaxR\_8i combine the principles of increased surface hydrophobicity with maximal Lys → Arg exchanges. They represent extremely fast NPC-shuttles.

	10	20	30	40	50	60	70	80	
EGFP	TSKGEELFTGVVPIVLVDGDVNGHKFSVSGEGEGDATYGKLTCLKFICTTGKLPVPWPTLVTTLTLYGVQCFSTRYPDHMKQ								80
MaxR_8M	.R.....R..R.....M..R.....R.....RR								80
2B7	.R.....R..R.....M..R.....R.....A.....RR								80
2B7A	.R.....R..R.....M..R.....R.....A.....RR								80
3B1	.R.....R..R.....M..R.....R.....A.....RR								80
3B7	.R.....R..R.....M..R.....R.....A.....RR								80
3B8	.R.....R..R.....M..R.....R.....A.....RR								80
3B9	.R.....R..R.....M..R.....R.....A.....RR								80
4B1	.R.....R..R.....M..R.....R.....A.....RR								80
3B7C	.R.....R..R.....M..R.....R.....A.....RR								80
7B3	.R.....R..R.....M..R.....R.....A.....RR								80
	90	100	110	120	130	140	150	160	
EGFP	HDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKEFGDTLVNRIELKGDIFKEDGNILGHKLEYNYNHNVYIMADKQKNG								160
MaxR_8M	.....M.Y.E...A.R...R.....R...R.....R...F...M.....R.R..								160
2B7	.....M.Y.E...A.R...R.....R...R.....R...F...M.....R.R..								160
2B7A	.....T.....M.Y.E...A.R...R.....R...R.....R...F...M.....R.R..								160
3B1	.....T.....M.Y.E...A.R...R.....R...R.....R...F...M.....R.R..								160
3B7	.....T.....M.Y.E...A.R...V.R.....R...R.....R...F...M.....R.R..								160
3B8	.....T.....M.Y.E...A.R...V.R.....R.T.R.....R...F...M.....R.R..								160
3B9	.....T.....M.Y.E...A.R...V.R.....R.G.R.....R...F...M.....R.R..								160
4B1	.....T.....M.Y.E...A.R...V.R.....R.R.R.....R...F...M.....R.R..								160
3B7C	.....T.....M.Y.E...A.R...V.R.....R...R.....R...F...M.....R.R..								160
7B3	.....T.....M.Y.E...A.R...V.R.....R...R.....R...F...M.....R.R..								160
	170	180	190	200	210	220	230		
EGFP	IKVNFKIRHNIEDGSVQLADHYQNTPIGDGPVLLPDNHYLSTQSALS KDPNEKRDHMLKEFVTAAGITLGMDELYK								238
MaxR_8M	.RAM.NT...V...E.V.M.....D...N.M.R..M...R.....R...M.....R								238
2B7	.RAM.NT...V...E.V.M.....D...Y.M.R..V...R.....R...M.....R								238
2B7A	.RAM.NT...V...E.V.M.....D...Y.M.R..V...R.....R...M.....R								238
3B1	.RAM.NT...V...E.V.M.....D...Y.M.L..V...R.....R...M.....R								238
3B7	.RAM.NT...V...E.V.M.....D...Y.M.L..V...R.....R...M.....R								238
3B8	.RAM.NT...V...E.V.M.....D...Y.M.L..V...R.....R...M.....R								238
3B9	.RAM.NT...V...E.V.M.....D...Y.M.L..V...R.....R...M.....R								238
4B1	.RAM.NT...V...E.V.M.....D...Y.M.L..V...R.....R...M.....R								238
3B7C	.RAM.NT...V...E.V.M.....D...Y.M.L..V...R.....R...M.....IR								238
7B3	.RAM.NT...V...E.V.M.....D...N.I.R..V...R.....R...M.....R								238

### Sequence alignment G: Engineering of GFP<sup>NTR</sup> variants of high FG-specificity and NPC-passage rate

The design principle was to place 8 ectopic (hydrophobic) methionines on the GFP's surface and to replace all exposed lysines by arginines. The essentially non-fluorescent initial MaxR\_8M mutant was evolved to highly fluorescent species. 2B7 crosses NPCs considerably faster than NTF2 (see Table 1), but still shows considerable non-specific interactions.

3B1-3B7C are *tetramers* of great FG- and NPC-specificity, with 3B7C being the most selective variant. 7B3 is a re-monomerized version of 3B7 and the fastest NPC-shuttle identified so far, showing less non-specific binding than 2B7.

	10	20	30	40	50	60	70	
drFP583	---MRSSKNV.....R..T.....HN.V.....Q...V.....	76						
mCherry	TSKGEEDNMAIIKEFMRFKVHMEGSVNGHEFEIEGEGEGRPYEGTQTAKLKVTKGGPLPFAWDILSPQFMYGSKAYVKHP	80						
tCherry	--T.MASSED.....R.....	78						
sin_tCherry1	--TS.ASSED....E.K..R.....K.....	78						
sin_tCherry2	--TS.ASSED....E.K..R.....K.....	78						
	80	90	100	110	120	130	140	150
drFP583	.....K.....C.....FI.V.....T..L..R..V	156						
mCherry	ADIPDYLLKLSFPEGFKWERVMNFEDGGVVTVTQDSSLQDGEFIYKVKLRGTNFPDGPVMQKKTMGWEASSERMYPEDGA	160						
tCherry	.....I.V.....T....R..V	158						
sin_tCherry1	.....Q.....E.....I.V.....E..Q....T....R..V	158						
sin_tCherry2	.....Q.....E.....I.V.....E..Q....T.H...R..V	158						
	160	170	180	190	200	210	220	
drFP583	.....HKA.....LV.F.SI.M.....Y.Y.DS.....T...HLFL	225						
mCherry	LKGEIKQRLKLDGGHYDAEVKTTYKAKKPVQLPGAYNVNIKLDITSHNEDYTIVEQYERAEGRHSTGGMDELYK	235						
tCherry	.....H.A.....L....I.M.....Y.Y.D.....HLFL	227						
sin_tCherry1	.....H.A.....L....I.Q....TE...Y.Y.D.....HLFL	227						
sin_tCherry2	.....H.A.....L....I.Q....TE...Y.Y.D.....HLFL	227						

**Sequence alignment H: Engineering of a super-inert tetrameric Cherry variant**

Starting point was mCherry (Shaner et al., 2004). tCherry is an mCherry derivative, where the tetramerisation interface of the original red fluorescent protein drFP583 had been restored. Sin\_tCherry1 is a hydrophilised version of tCherry. Sin\_tCherry2 carries an additional R149H exchange that improves the packing between the subunits, increases thermostability, and confers faster fluorophore maturation.



	10	20	30	40	50	60	70	80	
MBP	TKTEEGKLVIIWINGDKGYNGLAEVGGKFEKDTGIKVTVEHPDKLEEKFPQVAATGDGPDIIIFWAHDRFGGYAQSGLLAEI								80
MBP G261C	.....								80
MBP K→R	.R...R.....RR.R...R...R...R.....								80
	90	100	110	120	130	140	150	160	
MBP	TPDKAFQDKLYPFTWDAVRYNGKLIAYPIAVEALSIIYNKDLLPNPPKTWEEIPALDKELKAKGKSALMFNLQEPYFTWP								160
MBP G261C	.....								160
MBP K→R	...R...R.....R.....R.....R.....R...R.R.....								160
	170	180	190	200	210	220	230	240	
MBP	LIAADGGYAFKYENGGYDIKDVGVNAGAKAGLTFLVDLIKKNHMNADTDYSIAEAAFNKGETAMTINGPWAWSNIDTSK								240
MBP G261C	.....								240
MBP K→R	.....R...R...R.....R.....R.R.....R.....R.....R								240
	250	260	270	280	290	300	310	320	
MBP	VNYGVTVLPTFKGQPSKPFVGVLSAGINAASPNKELAKEFLENYLLTDEGLEAVNKDKPLGAVALKSYEEELAKDPRIAA								320
MBP G261C	.....C.....								320
MBP K→R	.....R.....C.....R.....R.....R.....R.....								320
	330	340	350	360	370				
MBP	TMENAQKGEIMPNIQMSAFWYAVRTAVINAASGRQTVDEALKDAQTN								371
MBP G261C	.....					N			368
MBP K→R	.....R.....					R		N	368

**Sequence alignment i: A K→R substituted mutant of the *E. coli* maltose-binding protein MBP, which crosses NPCs 40-fold faster than MBP itself**

Alignment shows the implemented substitutions. The G261C mutation creates an attachment site for a maleimide at the base of the maltose-binding groove. An introduced maleimide-fluorophore can thus at least partially be shielded from interactions with FG motifs. MBP K→R contains 29 lysine to arginine exchanges.

	10	20	30	40	50	60	70	80	
Imp $\beta$	MELITILEKTVSPDRLELEAAQKFLERAAVENLPTFLVELSRVLANPGNSQVARVAAGLQIKNSLTSKDPDIKAQYQQRW								80
Imp $\beta$ R→K	.....K.....K.....K.....K.....K.....K.....K.....K.....								80
Imp $\beta$ K→R	.....R.....R.....R.....R.....R.....R.....R.....R.....								80
	90	100	110	120	130	140	150	160	
Imp $\beta$	LAIDANARREVKNYVLQTLGTETYPSSASQCVAIGIACAIEIPVQWPELIPQLVANVTNPNSTEHMKESTLEAIGYICQD								160
Imp $\beta$ R→K	.....K.....K.....K.....K.....K.....K.....K.....K.....								160
Imp $\beta$ K→R	.....R.....R.....R.....R.....R.....R.....R.....R.....								160
	170	180	190	200	210	220	230	240	
Imp $\beta$	IDPEQLQDKSNEILTAAIQGMRKEEPSNNVLAATNALLNSLEFTKANFDKESERHFIMQVVCEATQCPDTRVRVAALQN								240
Imp $\beta$ R→K	.....K.....K.....K.....K.....K.....K.....K.....K.....								240
Imp $\beta$ K→R	.....R.....R.....R.....R.....R.....R.....R.....R.....								240
	250	260	270	280	290	300	310	320	
Imp $\beta$	LVKIMSLYYQYMETYMGPALFAITIEAMKSDIDEVALQGIEFWSNVCDEEMDLAIEASEAAEQGRPPEHTSKFYAKGALQ								320
Imp $\beta$ R→K	.....K.....K.....K.....K.....K.....K.....K.....K.....								320
Imp $\beta$ K→R	..R.....R.....R.....R.....R.....R.....R.....R.....								320
	330	340	350	360	370	380	390	400	
Imp $\beta$	YLVPILTQTLTKQDENDDDDWNPCKAAGVCLMLLATCCEDDIVPHVLPFIKEHIKNPDWRYRDAAVMAFGCILEGPEPS								400
Imp $\beta$ R→K	.....K.....K.....K.....K.....K.....K.....K.....K.....								400
Imp $\beta$ K→R	.....R.....R.....R.....R.....R.....R.....R.....R.....								400
	410	420	430	440	450	460	470	480	
Imp $\beta$	QLKPLVIQAMPTLIELMKDPSVVVRDTAAWTVGRICELLPEAAINDVYLAPLLQCLIEGLSAEPRVASNVCFWAFSSLAEA								480
Imp $\beta$ R→K	.....K.....K.....K.....K.....K.....K.....K.....K.....								480
Imp $\beta$ K→R	..R.....R.....R.....R.....R.....R.....R.....R.....								480
	490								
Imp $\beta$	AYEAADVADDQEE								493
Imp $\beta$ R→K	.....								493
Imp $\beta$ K→R	.....								493

### Sequence alignment J: K→R and R→K mutations of the human importin $\beta$ 1-493 fragment

The fragment was used because it expresses better and is more soluble than the full-length version. It also lacks the C-terminal cargo-binding region that would otherwise cause retention inside nuclei when influx is measured without Ran-addition. Figure shows alignment with the corresponding R→K mutant (16 substitutions) and the K→R mutant (16 substitutions).